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Modular Arrangement and Secretion of a Multidomain Serine Protease

EVIDENCE FOR INVOLVEMENT OF PROLINE-RICH REGION AND *N*-GLYCANS IN THE SECRETION PATHWAY*

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The *Limulus* Factor C (FC), a multidomain glycoprotein that binds bacterial endotoxin with high affinity, belongs to the serine protease family of the complement and blood coagulation cascade. Here, we provide compelling evidence for the importance of modular arrangement and relevance of the proline-rich region (PRR) and *N*-glycosylation to the secretion and function of FC. We propose that PRR could be a universal conformational domain that regulates protein folding and targeting. FCs lacking PRR preceding the serine protease domain, were localized intracellularly. Misfolded conformers of the intracellular FCs were more susceptible to trypsin digestion. Glycosylation inhibition studies indicate that the presence but not the exact structure of the *N*-glycans affects the secretion of FC, although the complexity of glycosylation may influence its endotoxin-induced proteolytic cleavage with resultant enzymatic activity. Disruption of specific *N*-glycan sites at positions 740, 767, and 912, downstream of the PRR, at or near the serine protease domain, blocks its secretion. Co-expressed molecular chaperones like canine calnexin associates with glycosylated FCs to increase its solubility and secretion level but did not alter their expression profiles. Our results clearly demonstrate that the folding and secretion of a multidomain serine protease like FC are determined by its modular domain arrangement and site-specific *N*-glycans. The secreted FCs containing the N-terminal portion of FC are able to detect lipopolysaccharide with high sensitivity. We also identified the lectin-like and sushi 4 domains to contribute to the binding of lipopolysaccharide.

The availability of engineered serine proteases allows the study of their activation, substrate specificity, and regulation of various physiological processes. As the initiator of the coagulation cascade of limulus amoebocyte lysate (LAL),[1] Factor C (FC) is a multidomain serine protease, which is activated by a trace amount of its ligand, endotoxin (lipopolysaccharide; LPS) (1). LAL is widely employed to detect the presence of LPS in various industrial and clinical products (2). Analysis of the amino acid sequence indicates that FC is a complex modular protein, comprising 11 domains (see Ref. 3 and Fig. 1*A*). Many earlier attempts to express FC in heterologous hosts have faced limited success (4–7). The native signal peptide of FC directs this molecule to intracellular large granules of the limulus amoebocytes, and it is unable to secrete the protein out of heterologous expression hosts. Instead, it may serve as a membrane anchor and invariably result in membrane-bound recombinant protein. Thus, an alternative signal peptide is needed to obtain secreted FC for investigation of its domain-function relationship. Using a cross-host secretory signal (8), which leads to the secretion of various recombinant proteins in both prokaryotic and eukaryotic systems, we have recently produced full-length and biologically functional domains of FC that binds LPS (9, 10).

The LPS binding and enzymatic activity of Factor C are functionally distinct and are localized in the N-terminal sushi (ES) and C-terminal serine protease domains (SP), respectively (10, 11). However, the importance of interdomain interactions for expression and biological activity of FC is hitherto unknown. Of particular interest is the proline-rich region (PRR) of the FC protein. Proline residues contribute significantly to the conformation and rigidity of the protein structure, and PRR serves as a molecular hinge in a number of proteins (12–14), affecting protein-protein interaction and protein-ligand binding. In this study, we focus on the potential involvement of PRR in FC secretion and function. By systematic design of FC deletion homologues with and without PRR, we are able to examine their expression profile, post-translational modifications, localization, and LPS binding activity.

N-Linked glycans play pivotal roles in protein folding, oligomerization, quality control, sorting, and transport (15). These sugar chains are extensively involved in protein conformation and function (16). There are six potential *N*-glycosylation sites in FC at positions 523, 534, 624, 740, 767, and 912

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[1] The abbreviations used are: LAL, *Limulus polyphemus* amoebocyte lysate; FC, Factor C; cCNX, canine calnexin; hCRT, human calreticulin; FL, full-length Factor C; ES, *Eco*47III-*Sal*I fragment of Factor C cDNA; SP, serine protease; PRR, proline-rich region; TM, tunicamycin; DMJ, α-deoxymannojirimycin; DNJ, α-deoxynojirimycin; LPS, lipopolysaccharide; SPR, surface plasmon resonance; CAL, *C. rotundicauda* amoebocyte lysate; S2 FC, full-length Factor C derived from the *Drosophila melanogaster* S2 expression system; Sf9-BEVS FC, full-length Factor C derived from *Spodoptera frugiperda*, Sf9-baculovirus expression vector system; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

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Seikagaku Corporation Exhibit 2015 - Page 1 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

(Fig. 1) (17). However, the functions of these glycans has not been examined. We used glycosylation inhibition and site-directed mutagenesis to test the role of *N*-glycans on expression and secretion of FC and to map the most critical *N*-glycans. Further assessment on the involvement of *N*-glycans in the secretion pathway was performed by co-expression of FC with various molecular chaperones, which are proteins that assist in the correct noncovalent assembly of other polypeptide-containing structures (18).

We report for the first time that expression and secretion of such a multidomain serine protease requires the proper modular arrangement of the various domains, and PRR is essential for proper folding of the downstream SP domain. In addition, we demonstrated the importance of site-specific *N*-glycosylation in FC secretion and provided evidence that *N*-glycans interact with chaperones and hence manifest their effect on FC secretion. Interestingly, despite having proper protein folding, the secreted FC exhibited LPS binding but lacked enzymatic activity. This loss of LPS-induced enzymatic activity can be attributed to different complexity in glycosylation.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all reagents were of molecular biology grade, purchased from Sigma.

Construction of Expression Plasmids

Drosophila expression vector, pAc5.1/V5-HisA (Invitrogen) was used for fusion expression. The native signal peptide sequence of full-length (FL) FC cDNA was replaced by a novel secretion signal to direct the fusion protein out of the *Drosophila* cells. Seven expression plasmids were constructed, with various combinations of FC domains inserted downstream of secretory signal and upstream of V5 and His_6 fusion tag: FL, FL(−PRR), ES-PRR, ES, PRR-SP, SP, and SP-PRR. Fig. 1 depicts the domain structures of these recombinant constructs. Among the seven constructs, FL, ES-PRR, PRR-SP, and SP-PRR contain PRR. The remaining three constructs (FL(−PRR), ES, and SP) lack PRR. Only ES, which contains the N-terminal LPS-binding region, lacks both PRR and *N*-glycan sites. Details on cloning strategies and secretory signal sequence are available upon request. Canine calnexin (cCNX) (19) and human calreticulin (hCRT) (20) cDNAs were subcloned into pAc5.1/V5-HisA.

Site-directed mutagenesis of the *N*-glycan sites of FC was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). Mutations were verified by sequencing.

Transient and Stable Transfections

Plasmids were isolated using CsCl-ethidium bromide gradient ultracentrifugation. *Drosophila* S2 cells were maintained at 25 °C in DES™ medium supplemented with 10% fetal bovine serum (CLONTECH). Calcium phosphate-mediated transient transfection was performed according to the manufacturer's recommendation (Invitrogen). For stable transfection, pCoHYGRO selection plasmid was cotransfected with Factor C expression construct, selected with 350 μg/ml hygromycin for 4–6 weeks, and adapted to DES™ serum-free medium.

For chaperone co-expression studies, Cellfectin-mediated (Invitrogen) transfection was performed with DNA mixture containing 1 μg each of FC and chaperone expression construct. Control transfections were performed with FC expression construct and parental pAc5.1/V5-HisA DNA instead of chaperone expression constructs.

Partial Tryptic Digestion of FC

Two μl of trypsin solution (100 ng/μl) in 50 mM acetic acid was added into 200 μg of FC in 100 μl of 50 mM NH_4HCO_3. The digestion was carried out at 4 °C. At time intervals, 10 μl of the reaction mixture was removed and terminated by 4 μl of the 4× SDS sample buffer. Western analysis was performed on the digested samples using polyclonal rabbit anti-FC antibody.

In Vivo Glycosylation Inhibition and ConA-Sepharose Binding Assay

Tunicamycin (TM) inhibits *N*-glycosylation of glycoproteins by blocking the first step in *N*-glycan biosynthesis, whereas α-deoxynojirimycin (DNJ) and α-deoxymannojirimycin (DMJ) specifically inhibit α-glucosidase and α-mannosidase activities, respectively, thus blocking the conversion of high mannose oligosaccharides to complex-type oligosaccharides. S2 cells stably expressing FC were exposed to sublethal doses of TM (5 μg/ml), DNJ (5 mM), and DMJ (2 mM). Proteins from treated cells were collected at 24 *h* and subjected to affinity chromatography on ConA-Sepharose (Amersham Biosciences). Conditioned medium containing 10 μg/ml secreted FC and cell lysate protein containing 1 μg/μl cellular FC were incubated with one-fifth volume of ConA-Sepharose at 25 °C for 1.5 h. The unbound fraction containing aglycosylated FC was recovered by centrifugation at 2000 × *g* for 5 min. The resin-bound glycosylated FC was dissociated via boiling for 5 *min* in 3 resin volumes of SDS-PAGE loading buffer. Both fractions were subjected to Western analysis.

Immunoprecipitation and Analysis of FC and Chaperone Association

S2 cells co-transfected with FC and chaperone expression plasmids were collected on day 5 post-transfection and washed twice with 1.0 ml of PBS. Cells were resuspended in 500 μl of lysis buffer (0.1× PBS, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 μM aprotinin), sheared seven times through a 27-gauge needle, and centrifuged at 14,000 × *g* for 15 min. Supernatant containing the cell lysate (L) proteins was retrieved. The pelleted membranes (P) were solubilized in 500 μl of lysis buffer containing 1% digitonin for 30 min at 4 °C with occasional agitation. The samples were clarified at 14,000 × *g* for 15 *min* to separate aggregates from solubilized proteins, which were used for subsequent immunoprecipitation.

Fifty μl of protein A-Sepharose (Amersham Biosciences) was incubated with 50 μl of PBS containing 2 μl of anti-V5 antibody for 1 h at 4 °C. After incubation, 100 μl of solubilized protein samples was immunoprecipitated at 4 °C overnight on a rotator. The samples were centrifuged and washed three times with cold PBS. Forty μl of SDS-PAGE loading buffer was added to each tube and boiled for 5 min before Western analysis.

Concentration and Partial Purification of FC

Culture medium containing secreted FC was collected after centrifugation of cell culture at 3000 × *g* for 10 *min* at 4 °C. Medium was concentrated 10-fold by ultrafiltration using a regenerated cellulose membrane cassette (Pellicon XL; 30-kDa molecular mass cut-off; Millipore Corp.) at 4 °C. Concentrated protein sample was chromatographed on a Sephadex G-100 (1.5 × 90-cm) column. Fractions containing partially purified FC were pooled and concentrated using Centriprep-regenerated cellulose membrane (30-kDa molecular mass cut-off; Amicon YM).

In Vitro Endoglycosidase Digestion and Glycan Differentiation

10 μg each of partially purified secreted FC from Sf9-BEVS and S2 was denatured in 5% SDS, 10% β-mercaptoethanol for 10 min at 100 °C and subjected to endoglycosidase H and peptide:*N*-glycanase F (New England Biolabs) digestion at 37 °C for 16 h, followed by Western analysis using anti-FC antibody. Control samples were treated in the same way except in the absence of enzyme.

For the glycan differentiation assay, FCs from LAL, CAL, Sf9-BEVS, and S2 were purified by protein A-FC antibody affinity chromatography. Fifty μl of protein A-Sepharose (Amersham Biosciences) was incubated with 50 μl of PBS containing 10 μl of anti-FC antibody for 1 h at 4 °C. 100 μg of FC samples were then added to the protein A-Sepharose complex and incubated at 4 °C overnight on a rotator. The samples were centrifuged and washed three times with PBS, and FC was recovered from protein A-Sepharose by boiling for 10 min in PBS. Lectin binding assay of the immunoaffinity-purified FCs was carried out using the DIG glycan differentiation kit (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Functional Analyses of FCs: Endotoxin Binding Assay

The LPS binding potential of various FC proteins were analyzed using (*a*) LPS ELISA-based assay and (*b*) surface plasmon resonance (SPR).

ELISA-based Assay for LPS Binding—This assay was performed as described by Tan *et al.* (10) with some modifications. Coating of a polysorp 96-well plate (NUNC) was achieved by incubation at 37 °C for 2 h, and blocking was performed overnight at 25 °C. 10 μg of protein sample was added into each well and incubated for 16 h at 25 °C. Bound FCs were detected by sequential incubation with primary rabbit anti-FC antibody (1:500 dilution, 3 h at 37 °C) and goat anti-rabbit secondary antibody (1:2000 dilution) conjugated to horseradish peroxidase (Dako).

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Seikagaku Corporation Exhibit 2015 - Page 2 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

SPR to Determine the K_d of FC for Lipid A—Anti-V5 antibody at 20 μg/ml was immobilized to CM5 chip (Amersham Biosciences) according to the manufacturer's specifications. A low capacity coating for kinetic study was achieved as indicated by an increase of 1418 resonance units. FC samples were injected at 10 μl/min over the anti-V5 antibody surface, resulting in capture of 5.0 pmol of FC (determined by densitometric scanning using pure ES as standard and normalized based on their peptide size) from the conditioned medium. Subsequently, lipid A at five different concentrations was injected at 20 μl/min to examine interaction between FC and lipid A. The affinity constant was calculated using BIAevaluation program (version 3.0.2) and reconfirmed using CLAMP (31). For regeneration, 100 mM NaOH was used. Values represent the mean of three independent experiments.

RESULTS

PRR Is Critical for the Correct Folding and Secretion of Downstream SP Domain—Seven expression plasmids were transfected into S2 cells. Immunoblot analysis shows that all the stable S2 transfectants expressed FCs, albeit displaying different amounts and expression profiles (Fig. 1*A*). Interestingly, although subcloned downstream of a potent secretion signal, not all of the FC proteins were secreted, suggesting that the localization of FCs might be signal sequence-independent. We observed that the FL could be detected in the culture medium. However, the deletion of PRR from FL, yielding FL(−PRR), is not secreted (Fig. 1*A*, *constructs 1* and *2*). This indicates that PRR is important for the secretion competency of FCs. We next investigated the relative contribution of PRR to the functionally distinct LPS-binding and serine protease domains, using paired truncated FCs (ES-PRR *versus* ES and PRR-SP *versus* SP) containing or lacking the PRR (Fig. 1*A*). Neither the preservation nor the removal of PRR affects the secretion competency of the LPS-binding domain, ES (ES-PRR and ES; Fig. 1*A*, *constructs 3* and *4*). Similarly, when PRR was localized upstream of SP (PRR-SP), as in the native FC, the PRR-SP protein was easily detectable in the medium. Interestingly, when the PRR was deleted, the serine protease domain alone (SP) is no longer detectable in the culture medium (Fig. 1*A*, *constructs 5* and *6*). This clearly indicates that PRR is critical for the secretion of the serine protease domain.

A major difference between secreted and cellular FCs that harbor SP was that the secreted FCs (FL and PRR-SP) have the PRR domain localized upstream of SP, without which SP is not secreted into the medium. To verify the importance of this modular arrangement between PRR and SP, the PRR was subcloned C-terminal to SP (SP-PRR). The PRR domain in this reciprocal construct was not able to rescue the intracellular retention of SP-PRR. Taken together, the PRR region of FC is important for correct folding of the downstream SP domain and that intracellularly localized FCs might be misfolded.

To shed more light on the role of PRR in folding of the downstream SP domain, partial tryptic digestion followed by Western analysis was carried out on two pairs of FCs: (*a*) FL and FL(-PRR) and (*b*) PRR-SP and SP. Over the observed time, distinct protein fragments of FL (132, 80, 52, and 38 kDa) and fragments of PRR-SP (56, 52, and 38 kDa) were clearly detectable by anti-FC antibody (Fig. 1*B*), suggesting that they were properly folded into a series of individual compact domains, which are relatively trypsin-resistant. It is interesting to note that two of the tryptic fragments, 80 and 52 kDa, corresponded to LPS-activated autocleavage of Factor C. Importantly, both PRR-deficient mutants, FL(−PRR) and SP, were rapidly digested by trypsin and were not detectable by anti-FC antibody after 1 h. Two isoforms of SP, of ~38 kDa, were observed, of which the larger isoform was rapidly digested by trypsin. Interestingly, the minor smaller isoform increased slightly over the tested time period. This increase could be generated from a small subpopulation of the larger isoform, and the smaller isoform was relatively resistant to trypsin digestion. These results show differences in protein folding between intracellular PRR-deficient FCs and secretion-competent FCs. It is envisaged that lack of PRR upstream of the SP domain tends to result in misfolded FCs, which expose more accessible trypsin cleavage sites that render them more susceptible to protease digestion.

Clearly, PRR is important for the folding and, hence, secretion of the serine protease. Essentially, PRR can only exert its effect when the serine protease domain is localized downstream of it.

N-Glycosylation Is Required for Proper Post-translational Processing—We also observed a differential dependence of the two functional domains (*viz.* LPS-binding and serine protease domains) on PRR for folding and secretion (Fig. 1). The secretion competence of LPS-binding ES is unaffected by PRR, which is in contrast to the SP. A distinguishing feature of ES is that it lacks *N*-glycosylation sites compared with SP or other SP-harboring FCs, which contain multiple *N*-glycosylation sites. Thus, we next investigated the significance of *N*-glycosylation on the expression and secretion of FC.

The secreted FL protein is glycosylated and is able to bind to ConA-Sepharose (data not shown). FL proteins from S2 cells treated with TM show progressively decreasing size based on their mobility (Fig. 2*A*), suggesting a loss of *N*-linked sugar chains. Aglycosylated FL was detected only in the L and P fractions, which clearly indicates that loss of *N*-glycosylation inhibited FL secretion. The absence of *N*-glycosylation of FL after TM treatment is also confirmed by its inability to bind ConA-Sepharose (Fig. 2*B*). In contrast to the effects of TM on FC secretion, cells treated with DNJ and DMJ exhibited no difference in expression level or distribution pattern of FCs (data not shown). Thus, the conversion of high mannose oligosaccharide to complex oligosaccharide is dispensable for protein secretion.

To determine the critical *N*-glycan sites, a series of glycomutations were introduced (Table I). The position of PRR is such that it partitioned the FL into the N-terminal LPS-binding and the C-terminal serine protease regions. Thus, we decided to mutate the *N*-glycosylation sites sequentially from each terminal. Single disruption of any one of the six *N*-glycan sites has no observable impact on the FC secretion (Fig. 2*C*, *Series I*). However, the elimination of two sites, particularly N523Q/N634Q and N767Q/N912Q, impaired the expression of FL (Fig. 2*C*, *lanes 1* and *5* of *Series II*). Although mutation of the three consecutive *N*-glycan sites located at the N terminus caused drastic reduction of the expression of FC, this mutant FL is still secreted into the culture medium (Fig. 2*C*, *lane 1* of *Series III*). In contrast and importantly, the mutation of the three consecutive C-terminal *N*-glycan sites located downstream of PRR at/near the SP domain totally abolished the secretion (Fig. 2*C*, *lane 2* of *Series III*). The removal of all six glycosylation sites (*i.e.* aglycosylated FL) is not secretion-competent (Fig. 2*C*, *Series IV*). Thus, in addition to PRR, the *N*-glycosylation sites in the SP are also critical for protein expression and secretion. We next sought to assess the contribution of *N*-glycans to secretion competence and also to determine a possible functional dependence on PRR.

N-Glycans Interact with Molecular Chaperones to Enhance Solubility and Secretion of FC—To further examine the involvement of *N*-glycans in the secretion pathway, co-expression study of FC with two molecular chaperones was carried out. cCNX and hCRT were expressed in S2 cells and their expected protein sizes and cellular localization were detected with their respective antibodies. The expression levels of the cCNX and hCRT in S2 were also comparable (Fig. 2*D*).

The overall expression level of FL, as determined by densi-

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Seikagaku Corporation Exhibit 2015 - Page 3 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
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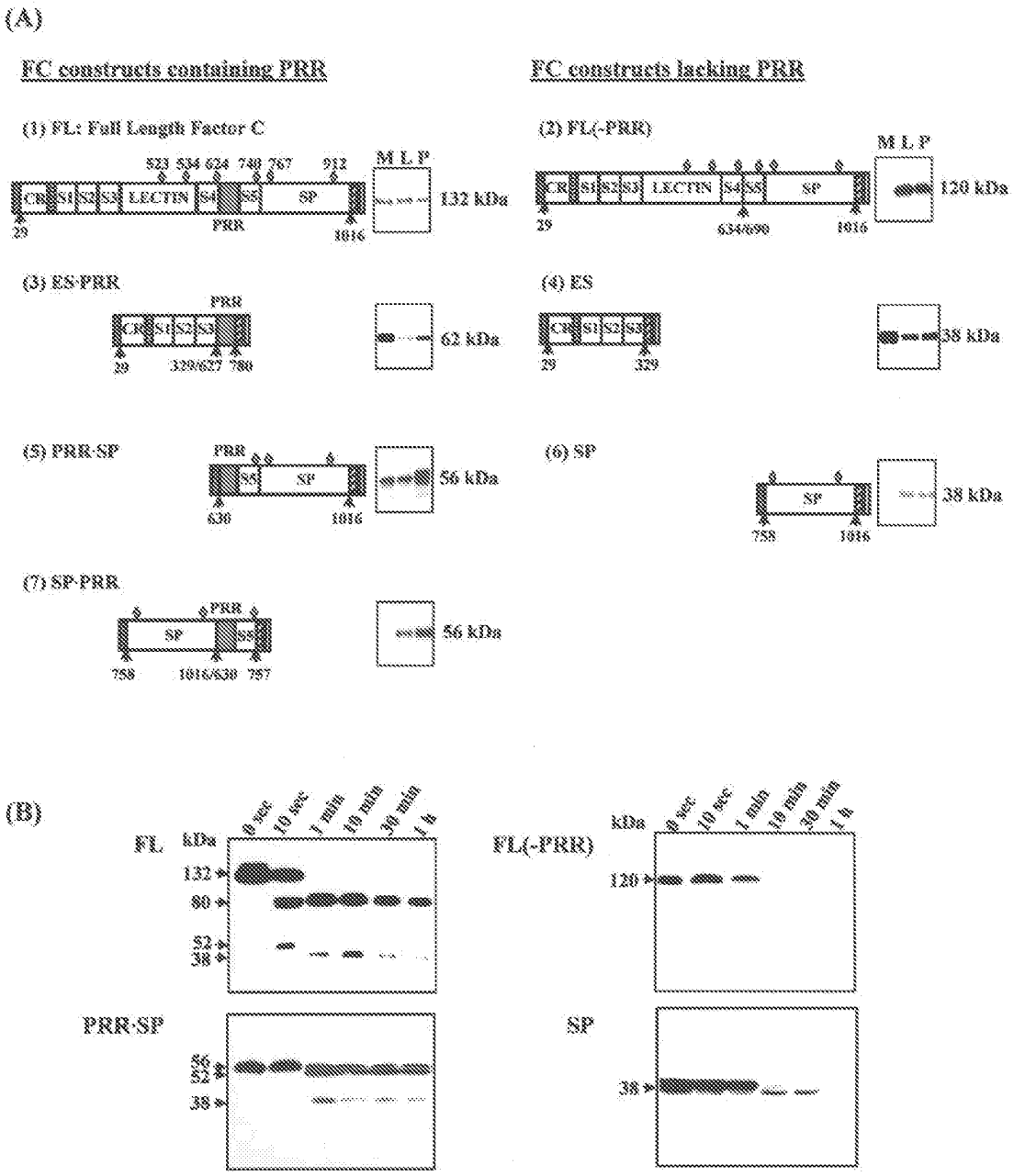

FIG. 1. **Domain structure, targeting, localization, and folding of FCs.** *A*, domain structure of the full-length Factor C and the deletion homologues. The recombinant fusion sites of different domains are indicated by *upward arrows* with the amino acid numbering in the original *Carcinoscorpius rotundicauda* FC21 (GenBank™ accession no. S77063). The glycosylation sites are marked with *green closed diamonds*. *Darkly shaded area*, secretory signal (*ss*); *CR*, cysteine-rich region; *horizontally striped area*, epidermal growth factor-like domain; *S1–5*, sushi-like domains 1–5; *LECTIN*, lectin-like domain; *PRR*, proline-rich region, in *red*; *SP*, serine protease coding region; *diagonally striped region*, V5 epitope; *vertically striped region*, His_6 tag. Targeting of the seven recombinant FCs was detected by Western blot. *M*, 1.0 μg of culture medium containing secreted proteins; *L*, 20 μg of cell lysates in PBS containing cellular proteins were obtained by five cycles of freeze-thawing of cells, followed by centrifugation at 12,000 × *g* for 15 min at 4 °C; *P*, 20 μg of insoluble pellet obtained at this step was also collected, weighed, and solubilized in 1× SDS-PAGE sample buffer. Protein concentration was measured by Bradford assay. The relative distribution of FC proteins in *M*, *L*, and *P* fractions was revealed using anti-V5-horseradish peroxidase antibody (Invitrogen). Membranes were visualized using the Supersignal® West Pico Chemiluminescent Substrate (Pierce). The amount of FC was quantitated densitometrically by using ImageMaster (Amersham Biosciences) software. Pure ES protein (10) was used as standard. Band intensities within the linear range were measured. *B*, partial proteolysis of FCs. FCs (FL, FL(-PRR), PRR-SP, and SP) were partially digested with trypsin and collected at time points as indicated. Digested samples were analyzed by Western analysis using polyclonal rabbit anti-FC antibody. Distinct stable protein fragments of FL (132, 80, 52, and 38 kDa) and PRR-SP (56, 52, and 38 kDa) were detectable.

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Seikagaku Corporation Exhibit 2015 - Page 4 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

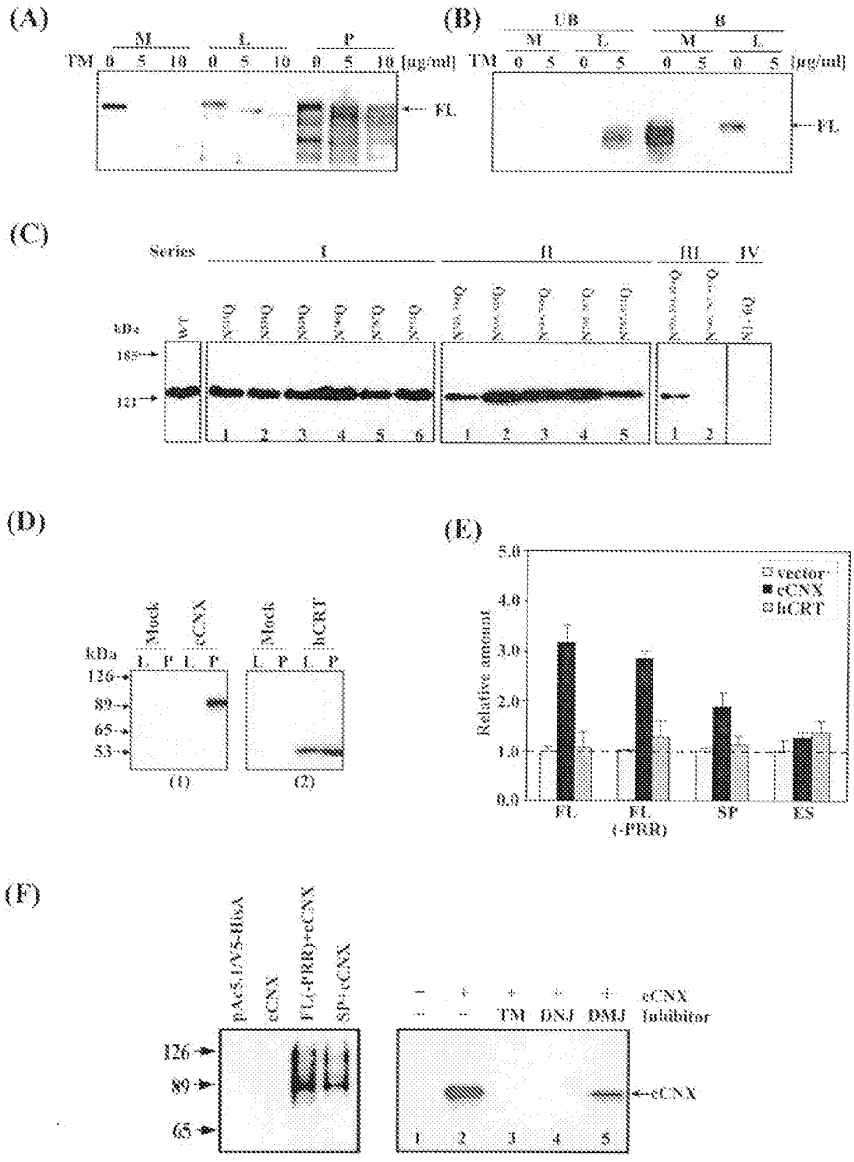

FIG. 2. ***N*-Glycans are required for proper FC synthesis and targeting.** *A*, immunoblot of FL from the M, L, and P after treatment of S2 cells with TM. Western analyses used 10 μg of medium (*M*), 20 μg of lysate (*L*), and 20 μg of solubilized pellet (*P*). *B*, immunoblot after ConA-Sepharose binding assay of FL from M and L. Protein samples were collected from cells treated or not with TM for 48 h. *UB*, unbound proteins; *B*, proteins bound to ConA-Sepharose. *C*, immunoblots of FC samples from S2 cells transfected with FC glycomutants. Cell culture supernatant was prepared from S2 expressing wild type FL and FL glycomutants. Protein samples were collected as described in legend of Fig. 1. 1.0 μg of medium was resolved in a 8% gel by SDS-PAGE. All transfections were done in parallel. The blot shown is representative of three independent experiments. Immunoblotting was performed with anti-V5-horseradish peroxidase antibody. *D*, expression level of FCs co-expressed with molecular chaperones. Expression of cCNX (*1*) and hCRT (*2*) in S2 cells is shown. S2 cells transfected with pAc5.1/V5-HisA vector or chaperone expression constructs were collected on *day* 5. Soluble lysate (*L*) and particulate (*P*) fractions were resolved by SDS-PAGE and analyzed using respective chaperone antibodies. Antibody for cCNX is from Transduction Laboratories, and antibody for hCRT is from Stressgen. cCNX is detected in the P fraction, and hCRT is detected in both the L and P fraction. These expression profiles conform to their original nature, in that cCNX is integrated into the ER membrane, whereas hCRT are ER luminal proteins. *E*, comparison of secreted FL, ES, and cellular FL(−PRR), SP co-expressed with chaperones on day 5 post-transfection. Relative values calculated from the densitometric data are shown, with the value for the S2 cells transfected with FC construct alone arbitrarily set as 1. *Error bars* represent the S.E. ($n = 3$). *F*, *left panel*, direct interaction of glycosylated FC with chaperones. Digitonin-solubilized cell lysates (*L*) from S2 cells transfected with vector-pAc5.1/V5-HisA, cCNX alone, FL(−PRR) + cCNX and SP + cCNX were precipitated overnight with anti-V5 antibody. The immunoprecipitates were subjected to SDS-PAGE and probed with anti-cCNX antibody. *Right panel*, association of chaperone and FC under glycosylation-inhibited conditions. S2 cells co-transfected with cCNX and SP (*lanes 2–5*) were pretreated overnight with TM (5 μg/ml, *lane 3*), DNJ (5 mM, *lane 4*), and DMJ (2 mM, *lane 5*) before transfection and incubated with these inhibitors after transfection. FCs expressed were then precipitated with anti-V5 antibody, and the chaperones that associated with FC were analyzed by SDS-PAGE and immunoblotting.

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Seikagaku Corporation Exhibit 2015 - Page 5 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

TABLE 1
Glycomutants of Factor C generated by site-directed mutagenesis

Mutant	Consensus sequence(s) disrupted
Series I FC mutants	
N523Q	Asn-523
N534Q	Asn-534
N624Q	Asn-624
N740Q	Asn-740
N767Q	Asn-767
N912Q	Asn-912
Series II FC mutants	
N523Q/N534Q	Asn-523 and Asn-534
N534Q/N624Q	Asn-534 and Asn-624
N624Q/N740Q	Asn-624 and Asn-740
N740Q/N767Q	Asn-740 and Asn-767
N767Q/N912Q	Asn-767 and Asn-912
Series III FC mutants	
N523Q/N534Q/N624Q	Asn-523, Asn-534, and Asn-624
N740Q/N767Q/N912Q	Asn-740, Asn-767, and Asn-912
Series IV FC mutant	
N1–6Q	All six sites

tometric scanning of the immunoblot, increased over time, and peaked between 5 and 7 days (data not shown). cCNX caused a 3-fold increase in the level of secreted FL, whereas hCRT minimally enhanced FL expression. Next, the effects of chaperone on the expression level of cellular FCs lacking PRR, FL(−PRR) and SP, were examined. Although overexpression of cCNX and hCRT enhanced the solubility of FL(−PRR) and SP to variable degrees (Fig. 2*E*), none of these chaperones was able to rescue the nonsecretion of PRR-deficient FCs: FL(−PRR) and SP. The chaperones did not affect ES expression and secretion. This is not surprising, since the LPS-binding ES domain is not glycosylated (Fig. 2*E*). Co-immunoprecipitation experiments showed that cCNX co-precipitated with FL(−PRR) and SP (Fig. 2*F*, *left panel*), but no apparent interaction was observed with hCRT (data not shown). Since hCRT consistently gives a small increase in FC expression, it is conceivable that the association between FC and hCRT is transient. Specific interaction between the *N*-glycans of FC and cCNX was further confirmed using *N*-glycosylation inhibitors, TM, DNJ, and DMJ. Consistent with the above observation, treatment with TM and DNJ abolished the interaction between FC and cCNX, whereas DMJ had no effect (Fig. 2*F*, *right panel*). This clearly indicates that the *N*-glycans on the FC molecules are responsible for interaction with cCNX. In addition, cCNX probably recognizes certain structures of FC *N*-glycans and binds specifically to the deglucosylated form of FC. Taken together, the results indicate that *N*-glycans are important for interaction with cCNX, which increases the solubility of the FC protein, and that its observed effect on protein secretion is secondary to the lack of this interaction. Furthermore, the PRR region, although critical for the correct conformation of the downstream serine protease domain, is dispensable for the interaction with these chaperones.

Specific Glycosylation Modification Is Required for FC Enzymatic Activity—In addition to its contribution to protein solubility, *N*-glycans have been reported to influence protein functions (16). Since glycosylation is the only post-translational modification known so far in FCs, we attempted to precisely investigate the extent and type of glycosylation in FC that can alter LPS-induced autocleavage and its enzymatic activity. Sf9-BEVS FC was previously demonstrated to exhibit LPS-induced cleavage and enzymatic activity (22).

Endoglycosidase H and peptide:*N*-glycanase F digestion analyses show the presence of both high mannose and complex glycans on S2 FC and Sf9-BEVS FC. Differences in their mobility indicate that Sf9-BEVS FC contains 12 kDa of complex *N*-glycans and S2 FC harbors 8 kDa of complex *N*-glycans (Fig. 3*A*). Thus, it appears that S2 FC is less glycolytically processed. *Galanthus nivalis* agglutinin treatment reveals the presence of high mannose glycans in all four FCs. *Sambucus nigra* agglutinin treatment yielded positive results for FCs from LAL, CAL, and Sf9-BEVS and only weakly reacted with S2 FC. Interestingly, reaction with *Maackia amurensis* agglutinin yielded the strongest signal with S2 FC, pointing to a difference in the branching of terminal sialic acids. S2 FC contain greater sialic acid linked α(2–3) to galactose, which is in contrast to Sf9-BEVS FC, LAL, and CAL. The latter three FCs contain more sialic acid linked α(2–6) to galactose. In agreement with the endoglycosidase H digestion results, *Datura stramonium* agglutinin yielded the weakest signal with S2 FC, confirming that S2 FC is the most poorly processed among the four FCs (Fig. 3*B*). Collectively, we have shown that the glycan structures of FC expressed in S2 are different from FCs derived from Sf9, CAL, and LAL. Such differences in glycomodifications can translate into disparity in protein functions.

LPS activation of the FC expressed in the Sf9-baculovirus expression system was cleaved into two protein fragments of 80 and 52 kDa (Fig. 3*C*), with resultant enzymatic activity (22). In contrast, attempts at measuring LPS-inducible enzymatic activity of S2 FC showed that it does not autocleave and exhibited no enzymatic activity. The above mentioned host cell-specific complexity of glycosylation of the FCs offers plausible explanations for this lack of cleavage and enzymatic activity.

Secreted FL and ES-PRR Retained Their LPS Binding Activities—Secreted S2 FCs were tested for their LPS binding efficacy using both ELISA and SPR. Using gel filtration-purified FCs, significant lipid A binding activity of FL and ES-PRR was observed, as compared with the partially purified conditioned serum-free medium from wild-type S2 cells. Both secreted FCs were capable of detecting as low as 0.05 μg/ml LPS (Fig. 4*A*). The secreted PRR-SP was unable to detect LPS, indicating that there is no LPS-binding domain in this molecule. The K_d values of FL and ES-PRR for binding to *E. coli* lipid A was determined using SPR. Fig. 4*B* shows a sensorgram of lipid A binding to FL. The K_d values were 2.23×10^{-7} M and 9.58×10^{-7} M for FL and ES-PRR, respectively. It is interesting to observe that the absence of the lectin-like and sushi 4 domains leads to a 4-fold decrease in affinity for LPS.

DISCUSSION

In this study, we have identified two important determinants of protein secretion of a mosaic serine protease: the PRR and certain strategically located *N*-glycans. PRR is necessary for proper folding of the downstream SP domain. The *N*-glycans, particularly those residing downstream of PRR, at or near the SP domain, contribute to stable interaction with specific chaperone(s) and consequently increase the solubility of FCs. Although both factors are involved in different aspects of protein expression, they are equally critical for the maturation and secretion of the protein (Fig. 5). We also identified two potential domains that contribute to the LPS binding activity of FC. The absence of the lectin-like and sushi 4 domain in SP-PRR leads to an apparent decrease in affinity for LPS by 4-fold compared with FL, indicating that these two domains could either affect the conformation of the upstream LPS binding domains or directly contribute to the binding of LPS. Finally, we provide evidence that the *N*-glycans are dispensable for LPS binding but that the differential complexity in glycosylation is critical for LPS-induced cleavage and enzymatic activity.

PRR Governs the Folding of Downstream SP Domain—The significance of modular arrangement to the structural integrity and functionality of multidomain proteins such as Factor XII and FC may provide us with important insights into their regulation, activation, and substrate specificity. The availabil-

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Seikagaku Corporation Exhibit 2015 - Page 6 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
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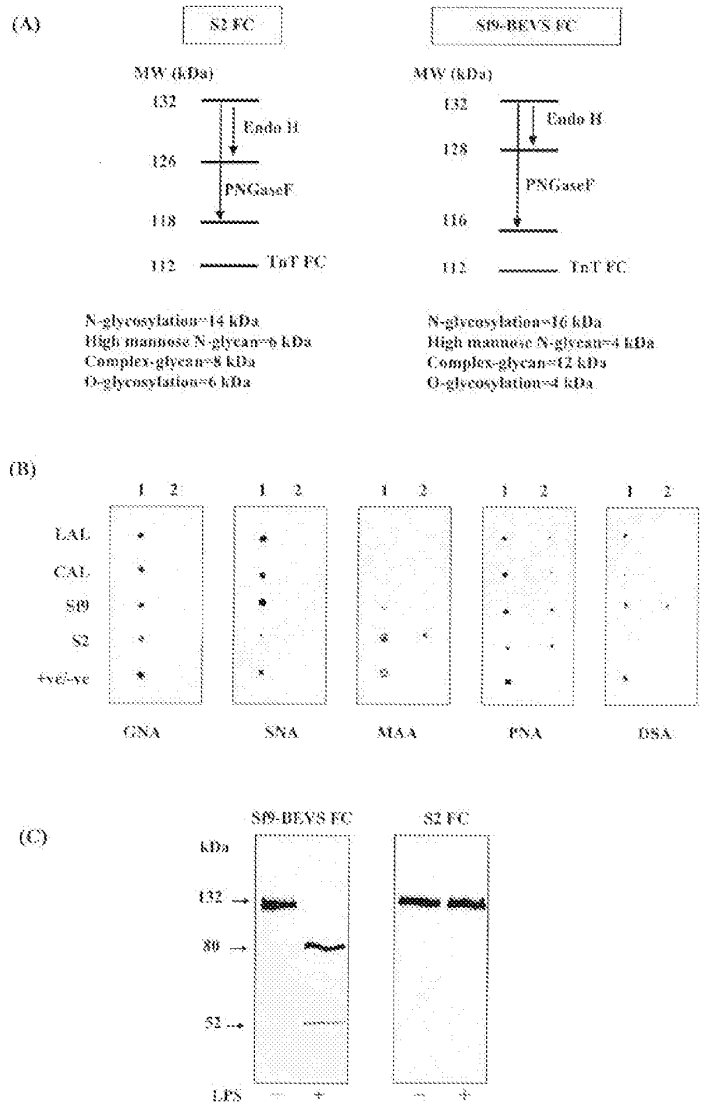

FIG. 3. **Comparison of the extent and complexity of glycosylation in S2 FC and Sf9-BEVS FC.** *A*, endoglycosidase treatment revealed that Sf9-BEVS FC has more complex *N*-glycans. The molecular mass shift after endoglycosidase H (*Endo H*) treatment indicates the extent of high mannose *N*-glycan chains, whereas the shift after peptide:*N*-glycanase F (*PNGase F*) treatment indicates total *N*-glycosylation (including both high mannose and complex sugar chains). The size difference between peptide:*N*-glycanase F-treated FC sample and FC TnT (transcription-coupled translation) product is attributable to *O*-glycosylation. *B*, glycan differential assay of S2 FC and Sf9-BEVS FC. *Lane 1*, FC; *lane 2*, FC without *N*-glycans. The *N*-glycans were detached from FC peptide chain by treatment with peptide:*N*-glycanase F, and the free glycan chains are removed by centrifugation through a 30-kDa molecular mass cut-off Microcon filter (Amicon). *G. nivalis* agglutinin (*GNA*), *S. nigra* agglutinin (*SNA*), *M. amurensis* agglutinin (*MAA*), peanut agglutinin (*PNA*), and *D. stramonium* agglutinin (*DSA*) have specific affinity for terminal mannose, sialic acid linked α(2–6) to galactose, sialic acid linked α(2–3) to galactose, *O*-glycosidically linked sugar chain, and Galβ-(1–4)GlcNAc in complex and hybrid *N*-glycans, respectively. *G. nivalis* agglutinin treatment yielded positive signals for all four FCs; *S. nigra* agglutinin treatment yielded positive results for FCs from LAL, CAL, and Sf9-BEVS and only weakly reacted with S2 FC. Reaction with *M. amurensis* agglutinin yielded the strongest signal with S2 FC. *S. nigra* agglutinin did not react with peptide:*N*-glycanase F-treated FC samples. *D. stramonium* agglutinin yielded the weakest signal with S2 FC. *C*, comparison of LPS activation of Sf9-BEVS FC and S2 FC. Incubation of 10 μg of Sf9-BEVS FC and S2 FC with 50 EU of LPS was carried out at 37 °C for 1 h. The activation of FC from Sf9-BEVS was readily observable (autocleavage into 80- and 52-kDa bands). S2 FC remained intact after the LPS treatment.

ity of secreted recombinant proteases in a heterologous system is usually preferable for ease of subsequent purification and functional studies. Since the secretion of a protein relies not only on its signal peptide but also on the intrinsic amino acid sequence that determines the final tertiary structure of the mature protein, we examined the secretion of FC constructs containing different modular domains in their native and reciprocal positions.

In the middle of the FC molecule is the basic PRR (amino acids ^{641}QNPPVPSYGSVEIKPPSRTNSISRVGSPFLRL-PRLPLPLARAAKPPPKPR^{690}), which contains three Src homology 3 binding core motifs (23), PXXP (underlined; P denotes proline). Src homology 3 domains affect protein targeting and enzymatic activity and play a role in signal transduction (24). It is possible that the PRR of Factor C is involved in transducing LPS-induced activation of the serine protease enzymatic activity as well as contributing to the LPS-dependent multiactivation of Factor C via protein-protein interaction (25). In addition, the PRR of Factor C shares homology with the hinge region of human coagulation Factor XII (26). Hence, it is possible that the PRR acts as a crucial conformational motif necessary for the secretion and function of such serine proteases.

In this study, we constructed and expressed a pair of FCs (FL and FL(−PRR)) with a view to examine the functional significance of PRR. The FL was efficiently secreted and fully capable of binding LPS. In contrast, the FL(−PRR), which lack the PRR, were expressed intracellularly and were nonfunctional. The expression profile of three additional FCs, SP, PRR-SP, and SP-PRR, confirmed that PRR is essential for folding and secretion of the downstream SP domain. The introduction of PRR C-terminal to ES has no observable effect on ES expression profile, suggesting that PRR only exerts its influence on the downstream domains. Thus, PRR itself may act as an intramolecular chaperone for correct folding of the downstream SP region for its subsequent transportation through the organelle. In support of our data, it was reported that the mini-

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Seikagaku Corporation Exhibit 2015 - Page 7 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
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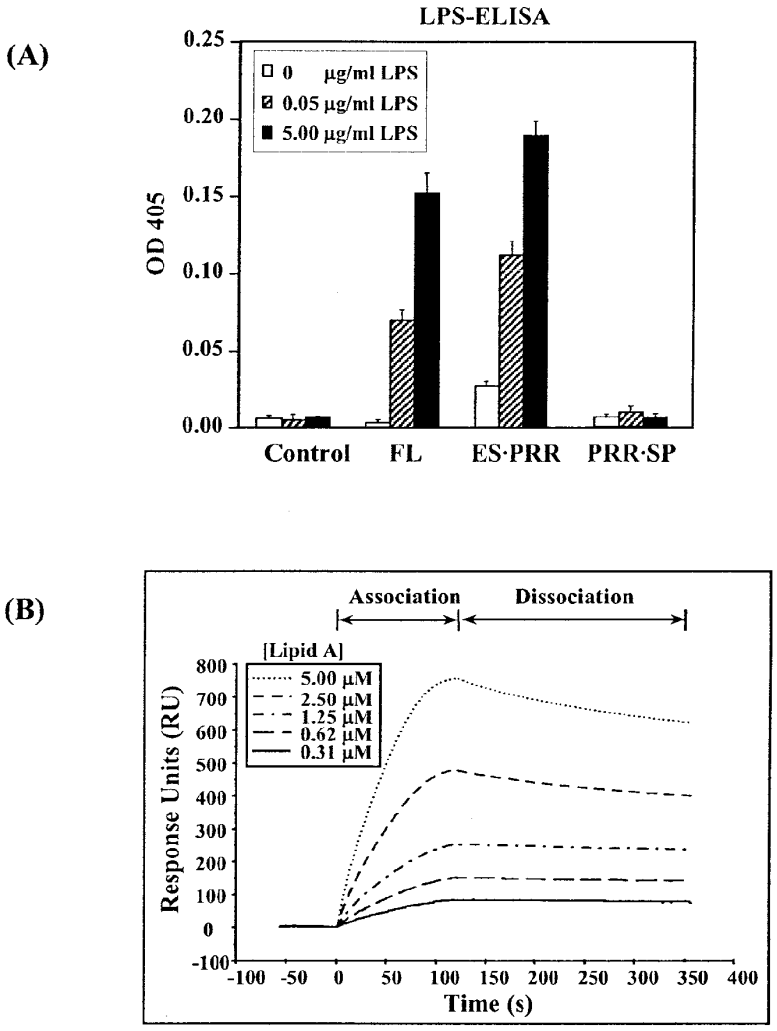

FIG. 4. **Functional analysis of FC.** *A*, ligand binding assays of FC samples. ELISA of 10 μg each of protein samples: control serum-free medium, FL, ES·PRR, and PRR·SP partially purified through Sephadex G-100 (void fraction). Three concentrations of LPS (0, 0.05, and 5 μg/ml) were used. *Error bars* represent the S.E. ($n = 3$). *B*, a sensorgram depicting the interaction (in response units (*RU*)) of captured FL with various concentrations of lipid A. ES·PRR gave similar response profiles. 100 μl of five concentrations (5.00, 2.50, 1.25, 0.62, and 0.31 μM) of lipid A were injected. The affinity constants were calculated using the BIAevaluation program version 3.0.2 and reconfirmed using CLAMP (21).

mal portion of secreted Factor XII, which is also a multidomain serine protease similar to FC, also contains PRR (27).

The differential susceptibility to trypsin clearly demonstrates the importance of PRR in the correct folding of the downstream SP region and in directing the secretion of glycosylated FC (Fig. 1*B*). Trypsin acts preferentially at the unfolded regions in proteins, such as the domain boundaries of a mosaic protein. It is worth noting that on the basis of sequence specificity alone, there are close to 100 and 50 potential trypsin cleavage sites for FL and PRR·SP, respectively. However, only three or four characteristic bands, representing two sets of relatively protease-resistant fragments, were detected after partial tryptic digestion. Therefore, the stable fragments are attributable to the compactly folded individual domains. The 38-kDa stable fragment observed after digestion of both FL and PRR·SP could be the properly folded SP domain. On the contrary, PRR-deficient mutants, FL(−PRR) and SP, failed to give rise to characteristic patterns of stable products, suggesting that they are incorrectly folded and are more susceptible to proteolytic digestion.

The crystal structure of a catalytic fragment from human C1s, which shares high similarity to Factor C modular architecture, has been determined (28). The CCP2 module (sushi domain) of C1s is oriented perpendicularly to the surface of the SP domain, and the connection is maintained through a rigid module-domain interface involving intertwined proline- and tyrosine-rich polypeptide segments (28). Since the residues maintaining the interface framework are highly conserved in the sushi-SP family, it is believed that the rigid sushi-SP assembly is also present in Factor C (29). Consistent with the role of a module-domain interface, our data show that the absence of PRR changes the sushi-SP assembly, resulting in misfolding of the deletion homologues, rendering them nonsecretory.

Many other studies have documented the importance of PRR to the biological functions of different proteins, and that its removal adversely affects the localization and activity of such proteins (30–32). Thus, rather than being restricted to a small subset of serine proteases, the PRR could be a universal conformational motif that regulates protein targeting.

N-Glycans Contribute to the Interaction with Chaperones and Solubility of the Recombinant Proteins—Whereas the data above clearly demonstrate that PRR is an important secretory determinant of FC, PRR also exerts a preferential effect on the

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Seikagaku Corporation Exhibit 2015 - Page 8 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
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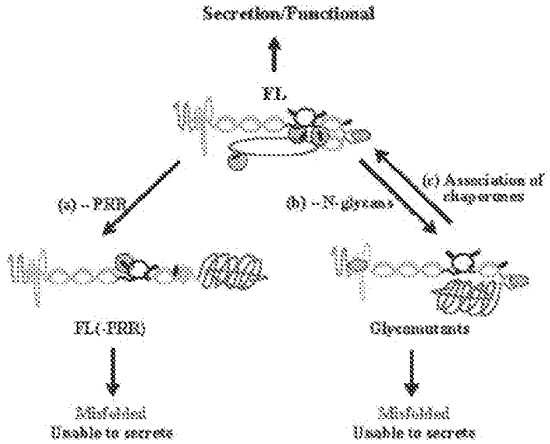

FIG. 5. **An illustration of PRR and *N*-glycans involved in secretion of FC.** *Gray string*, cysteine-rich region; *orange string*, epidermal growth factor-like domain; *green string*, sushi-like domains; *black circle*, lectin-like domain; *red string*, proline-rich region; *blue oval*, serine protease native conformer; *blue string*, misfolded serine protease; *black oval*, *N*-glycan; *round gray shapes*, proteins involved in folding, assembly, and secretion (*e.g.* chaperones, foldases, signal peptidase, and glycosidase). The hypothesis was confirmed by (*a*) the removal of PRR from FL, which completely switches its expression profile and protein conformation and reconfirms PRR to be an important secretory determinant for glycosylated FC; (*b*) mutation of *N*-glycan sites that impaired the secretion competency of FC; and (*c*) co-expressed chaperone (*e.g.* calnexin), which associates with glycosylated FL protein and assists the reversal of this impairment to a certain extent, to enhance its solubility and secretion. Thus, whereas PRR is crucial for correctly directing folding and secretion of the downstream glycosylated SP domain, chaperones and foldases also play a role in enhancing protein folding, to ensure that the modular arrangement of the glycosylated protein is properly folded to remain soluble or targeted for secretion and to exhibit full functionality.

serine protease domain, SP, and not on the LPS-binding ES domain. A distinctive characteristic is that ES does not harbor any *N*-glycosylation sites. Using specific glycosylation inhibitors and site-directed mutagenesis, we demonstrate that inhibition of glycosylation blocked the secretion of FL. The aglycosylated FL became intracellularly localized although their corresponding PRR was present, thus indicating that *N*-glycosylation is also critical for PRR-dependent secretion (Fig. 5). The importance of *N*-glycans for the secretion of FC was recapitulated using site-directed mutagenesis of the *N*-glycosylation sites. Interestingly, the ablation of the three *N*-glycan sites at the C terminus at positions Asn^{740}, Asn^{767}, and Asn^{912} completely abolished secretion. Coincidentally, these three sites reside downstream of PRR at or near the SP domain. The partial dependence of FC secretion on *N*-linked oligosaccharide appendices suggests that the individual *N*-glycans do not have local effects on protein folding. However, when the *N*-glycosylation sites at positions 740, 767, and 912 are modified at the same time, folding and secretion of FC are compromised although none of the glycans are needed individually. In view of this observation, the oligosaccharides of FC seem to have a global effect on the folding/secretion process.

These data suggest that correct folding of the SP domain is the most critical for secretion of FC; the absence of PRR preceding this domain or the absence of *N*-glycans within this domain leads to misfolding, which impairs FC secretion.

The mechanism by which *N*-glycans aid in FC secretion is attributable to its ability to interact with chaperones. Overexpression of chaperones has been proven to improve protein folding and stimulate secretion of certain target proteins (34–37). In this study, the secretion of FL and solubility of the cellular glycosylated FCs (FL(–PRR) and SP) were greatly enhanced by the presence of cCNX. Specific interaction between certain structures of *N*-glycans and calnexin was further confirmed by a glycosylation inhibition study. Thus, it is evident that the *N*-glycans of FC function through interaction with molecular chaperones to aid in protein folding and subsequent secretion. On the other hand, co-expressed chaperones did not overcome the nonsecretory problem of FL(–PRR) and SP, suggesting that their intracellular localization was determined by their conformation and not the lack of certain molecular chaperones in the host cell secretion pathway.

Functional FC should display both LPS binding activity and LPS-activated enzymatic activity. However, these two properties were uncoupled in S2-produced FC. S2 FC binds LPS, but it is not cleaved and not enzymatically activated. This is in contrast to Sf9-BEVS FC, which we demonstrated previously to harbor both functions (22). Further analyses reveal that the loss/gain of enzymatic activity stems from subtle differences in the complexity of glycosylation of FC. In agreement with our results, over the last decade, various reports (38–41) have described that viral infection of Sf9 cells temporally effects protein glycosylation (*i.e.* the activation of glycosyltransferase genes takes place under proper conditions, depending on the virus type, promoter, and medium). Normal glycosylation characteristics of insect cells were thus altered to allow complex type oligosaccharide processing to occur. Thus, during baculoviral infection of Sf9 cells for expression of FC, the viral infection process affects the glycosylation of FC, consequently allowing its conformational change upon LPS binding, leading to limited proteolysis and activation of the serine protease enzyme. In contrast, being nonvirally infected, the S2 FC bears differently processed glycans, especially those at/near the catalytic domain. Thus, due to the nonnative branching of the adjacent *N*-glycans, the S2 FC is not proteolytically cleaved, although it possesses LPS binding properties. This is despite the correct overall protein folding and successful passage of the S2 FC through the quality control of the ER and Golgi, which allows its successful secretion. However, since FC is such a demanding glycoprotein, retaining the LPS binding activity in these secreted FCs does not satisfy the requirement for their conformational change induced by LPS binding to yield enzymatic activity. This reveals another surprising impact of *N*-glycans on FC functions. Although differences in recombinant protein localization (42) and *N*-glycosylation heterogeneity (43) have been reported in these two insect cell expression systems, FC from these two hosts is so far the first recombinant protein to exhibit such marked functional difference. Future understanding and manipulations in the post-translational processing pathway with exoglycosidase/glycotransferases could possibly resolve this problem faced by FC as well as other serine proteases.

In summary, we confirm that PRR of a serine protease is essential for the folding and secretion of the downstream catalytic domain. These results suggest that the PRR serves a simple but essential role in tethering the structurally distinct but interacting domains of the protein. In addition, the secretion ability of FCs was impaired in the absence of *N*-glycans, especially those residing at or near the catalytic domain. We also report for the first time that co-expression of chaperones significantly increases the soluble and secreted recombinant protein level in S2 cells but has no appreciable effect on the localization of the protein. These results help to elucidate the function of different structural domains of Factor C and how the intrinsic properties of various domains of such a mosaic protein influence its destination (illustrated in Fig. 5). This

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Seikagaku Corporation Exhibit 2015 - Page 9 of 10
Charles River Laboratories, Inc. v. Seikagaku Corporation
IPR2025-00440

general concept of the modular arrangement may be applicable to other members of the multidomain protein family, such as blood coagulation Factor XII, in particular the influence of domain interactions on their cell sorting, secretion, and function. Finally, PRR is therefore a potential universal conformational domain that regulates protein folding and secretion, and it is probably applicable as domain fusion junctions for engineered chimeric multidomain proteins expressed in eukaryotic expression systems.

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Charles River Laboratories, Inc. v. Seikagaku Corporation
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